IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.: 10/554,291

Applicant: **Roberto Tonelli et al.**

Filed: September 18, 2006

Docket No.: BUG5-38919

DECLARATION OF ROBERTO TONELLI

1. I presently reside at via dello Sport, 19 – 40057 – Granarolo dell'Emilia – Bologna - Italy.

2. I am professor of Pharmacology at University of Bologna since October 2008 and the current inventor of the above-referenced U.S. Patent Application Serial No. 10/554,291, filed September 18, 2006.

3. My educational background is as follows. I received a degree in Biological Sciences by the University of Bologna in 1994, PhD Degree in Hematology by the University of Modena in 1999, and then post-Doc assistant professor in Pediatric Oncology. I published 32 scientific articles in peer-reviewed journals in the field of pharmacology and more than 50 abstracts in the scientific field.

4. Certain tests were conducted under my supervision with respect to the invention described in the above-referenced U.S. Pat. App. No. 10/554,291.

The present experimental data regard the ability of additional sense (anti-*N-myc*) PNA molecules to down-regulate *N-myc* expression. These experiments were performed in order to demonstrate that claim 1 is commensurate in scope. The impairment of *N-myc* gene expression induced by said sense anti-*N-myc* PNAs has been assessed by *N-myc* mRNA quantification by real-time PCR analysis, while the biological effects of sense anti-*N-myc* PNAs administration has been evaluated by cell growth inhibition assays. The assays are described as follows.

METHODS

PNA sequences

Anti-gene PNAs targeting specifically unique sequences in the antisense strand of the human *N-myc* gene, were designed and synthesized to block its transcription. These PNAs are scattered all along N-myc gene.

In particular, new PNA molecules were conjugated to a nuclear localization signal

peptide (NLS) Pro-Lys-Lys-Lys-Arg-Lys-Val.

The PNA specific sequences used in these experiments are the following:

PNA-1:	**GACAGTCATCTGTCTG**	**PNA-2:**	**CCTGTCGTAGACAGCT**
PNA-3	**CTCGAGTTTGACTCGC**	**PNA-4**	**CGTCGATTTCTTCCTC**

Cells

Kelly neuroblastoma cell line, characterized by *N-myc* gene amplification, and thus over-expressing *N-myc*, was used to test PNA molecules.

Phoenix cells were used as control cells. These cells are human fibroblasts that do not show *N-myc* gene amplification.

Real-time RT-PCR

1. $1.0x10^5$ cells per well were seeded onto 24-well cluster plates, with 0.5 ml of OPTI-MEM (GIBCO BRL) medium containing 4% FCS, 2mM L-glutamine and 1% penicillin/streptomycin (triplicate experiments).
2. Cells were incubated for 12h at 37°C, 5% CO_2 to allow cell adhesion.
3. Each anti-*N-myc* PNA was added at concentrations of 20 μM.
4. Cells were treated 12h with anti-*N-myc* PNA and then total cell RNA was purified using the RNeasy Mini Kit (QIAGEN).
5. Untreated cells were used as control.
6. Each RNA sample was quantified twice with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).
7. First-strand cDNA was synthesized by using cDNA Synthesis Kit for RT-PCR (Roche).
8. 1 μg of total RNA was used for cDNA synthesis.
9. 10 ng of cDNA in a final volume of 20 μl was used to performed real-time PCR (triplicate experiments) by using the SYBR Green Master Mix 2X (Applied Biosystems) (3 identical experiments).

10. The sequences and the concentration of the primers were:

Primer name	Sequence	Concentration
MYCN sense	CGACCACAAGGCCCTCAGT	300 nM
MYCN antisense	TGACCACGTCGATTTCTTCCT	300 nM
ACTB sense	GAGCACAGAGCCTCGCCTTTG	300 nM
ACTB antisense	ACCATCACGCCCTGGTGCCTG	300 nM
BIRC4 sense	ACAAGGAGCAGCTTGCAAGA	300 nM
BIRC4 antisense	AGCATGTTGTTCCCAAGGGT	300 nM

11. QRT-PCR reaction conditions were 2 min at 50°C, 10 min at 95°C, 15 sec at 95°C, and 60 sec at 60°C for 40 cycles.

Cell-Growth Assay

1. Kelly neuroblastoma cells and Phoenix cells were cultured as previously described.
2. Phoenix cells are used as control cells, since these cells do not show *N-myc* amplification/over-expression.
3. The Anti-*N-myc* PNA concentration that was administered to cells was 20 μM.
4. Cells were harvested and counted 72h after PNA administration.
5. Cell count and viability were determined using the ATPlite assay (Luminescence ATP Detection Assay System, PerkinElmer).
6. $5x10^3$ cells were seeded onto 96-well cluster plate (triplicate experiments have been done) with 100 μl of OPTI-MEM (GIBCO BRL) medium containing 4% FCS, 2mM L-glutamine and 1% penicillin/streptomycin.
7. Cells were processed according to the kit instructions after an incubation step performed for 12h at 37°C, 5% CO2 for allowing cell adhesion.

RESULTS AND DISCUSSION

The results of the experiments above reported, performed by using Kelly cells that, as said before, are neuroblastoma cells characterized by *N-myc* gene amplification and thus *N-myc* over-expression, demonstrate that all anti-*N-myc* PNAs assayed caused specific inhibition of *N-myc* transcription (see figure 1).

The specificity of the inhibition of *N-myc* gene transcription has been demonstrated by assaying anti-*N-myc* PNA molecules in Phoenix control cells wherein no effect on *N-myc* gene expression has been detected (Phoenix cells do not show *N-myc* gene amplification - see figure 3).

N-myc transcription impairment, induced by anti-*N-myc* PNAs, was followed by the specific cell-growth inhibition of Kelly cells (see figure 2). Indeed, it has been demonstrated that Kelly cells stop to proliferate after PNA (20 μM) administration; on the contrary, untreated cells did not show any cell growth inhibition.
Anti-*N-myc* PNAs were administered to Phoenix control cells to demonstrate that cell proliferation impairment was a consequence of the specific inhibition of *N-myc* gene expression (see figure 4).

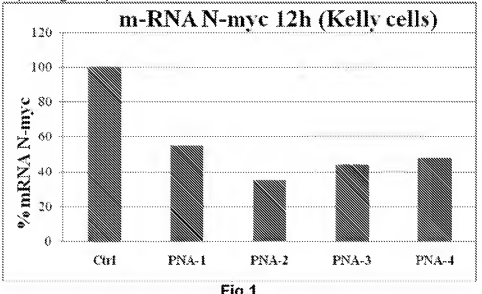

Fig.1

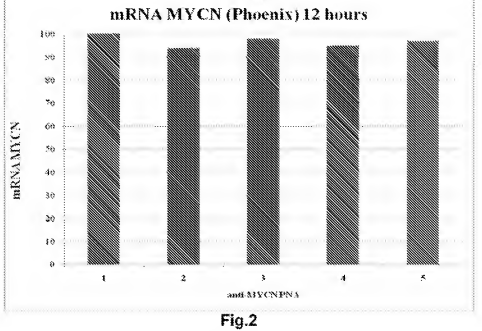

Fig.2

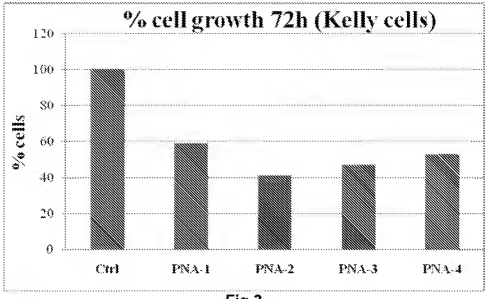

Fig.3

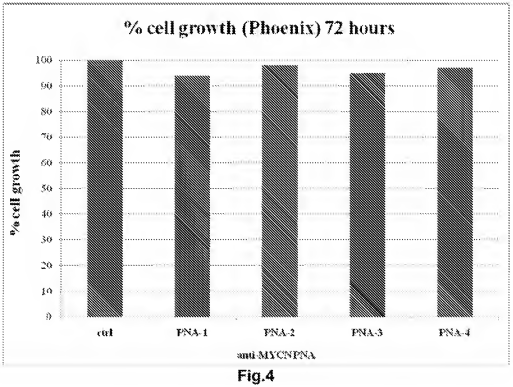

Fig.4

The pending patent Application demonstrated that the anti-*N-myc* PNA **ATGCCGGGCATGATCT (SEQ ID NO: 3)** is able to impair N-myc gene expression and to induce the death of cells over-expressing *N-myc* gene.

The results I presented herewith demonstrate that also further anti-*N-myc* PNA molecules, complementary to anti-sense strand of *N-myc* gene and scattered all along

the gene, are able to block specifically *N-myc* expression in biological models wherein *N-myc* is over-expressed.
Moreover, said gene expression impairment, causes the block of cell proliferation and induces cell death.

These further anti-*N-myc* PNA molecules contain 12-24 nucleotides. Moreover said PNAs are complementary to anti-sense strand of *N-myc* gene, and thus fall within the scope of claim 1.

In view of these additional experimental evidence I believe that claim 1 is commensurate in scope.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the patent application or any patent issued thereon.

August 6, 2010

Date

ROBERTO TONELLI